Validation of CRISPR/Cas9 Off-Target Discovery Profiles from *in Silico* Prediction, Cell-Based and Biochemical-Based Assays with Targeted Off-Target Sequencing

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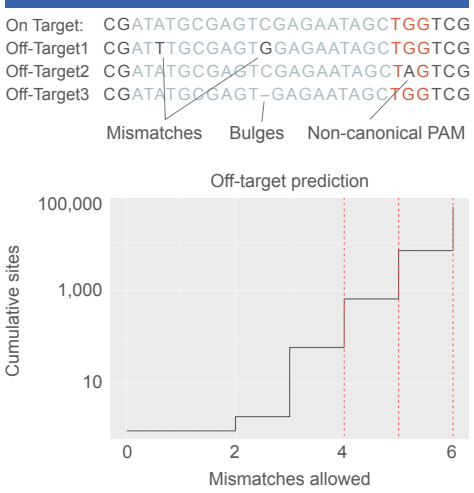
ABSTRACT

Therapeutic development of CRISPR/Cas9-based drug candidates relies on the identification of specific guide RNAs (gRNAs) that precisely determine the chromosomal location for introducing the gene edit, to alter, introduce or remove genetic information. We have developed a comprehensive workflow that applies a combination of both *in silico* and empirical approaches, using genome-wide assays and targeted sequencing, to identify and verify candidate gRNA off-target sites. Our *in silico* algorithm predicts potential off target sites by allowing three mismatches in the genome and four mismatches in coding DNA sequence. Empirical approaches for off-target site identification are the biochemical discovery assay *SITE-Seq*® and the cell-based oligo capture assay GUIDE-Seq. We present a case study based on 12 SpCas9 gRNAs designed for gene knockout. All candidate off-target sites identified through empirical approaches, plus the top 30 *in silico* predicted loci were experimentally interrogated through targeted off-target sequencing. Our data revealed that *in silico*-based methods had the lowest contribution to validated off-target discovery. The biochemical discovery assay *SITE-Seq*® was the most sensitive and identified validated off target indels that were missed by the cell-based GUIDE-Seq assay. Based on these results and our internal database, we favor an integrative genomics approach that applies an *in silico* prediction algorithm and an empirical biochemical off-target discovery assay such as *SITE-Seq*®, for identification of potential off target sites to be further validated by targeted off-target sequencing. This workflow supports the selection of gRNAs with the specificity and precision required for CRISPR/Cas9 drug development.

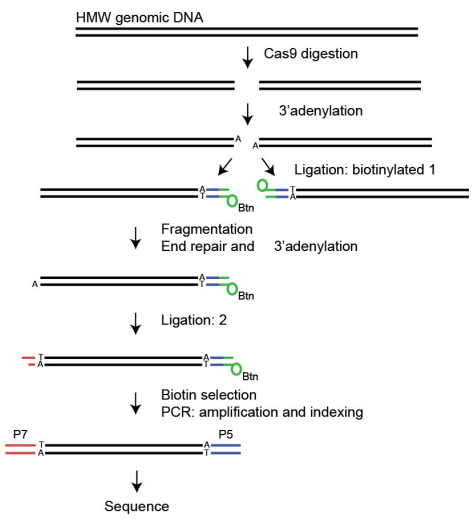
IN SILICO OFF-TARGET DISCOVERY



In Silico Site Prediction Based on Mismatches

The cumulative number of sites predicted is represented on the Y-axis. There is log-linear relationship in the number of potential off-target loci as the number of mismatches allowed increases (X-axis).

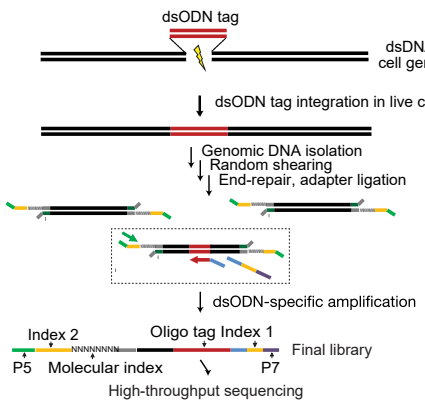
BIOCHEMICAL[1] OFF-TARGET DISCOVERY



SITE-Seq® NGS library construction

This is a species genome-wide off-target discovery assay because it is executed on deproteinated and purified genomic DNA.

CELL-BASED[2] OFF-TARGET DISCOVERY

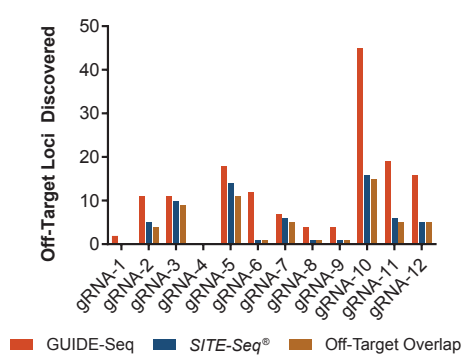


GUIDE-Seq NGS Library Construction

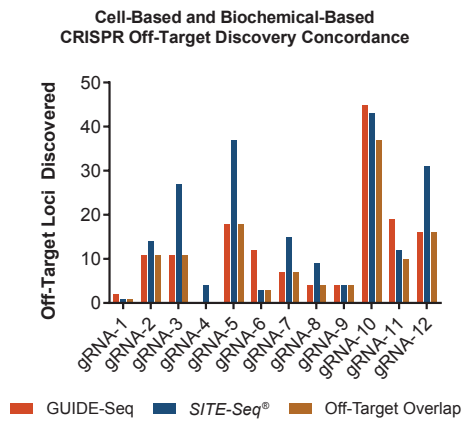
This is a tissue specific genome-wide off-target discovery assay because it is executed in cells and relies on endogenous repair machinery.

BIOCHEMICAL VERSUS CELL-BASED AND *IN SILICO* OFF-TARGET PROFILES

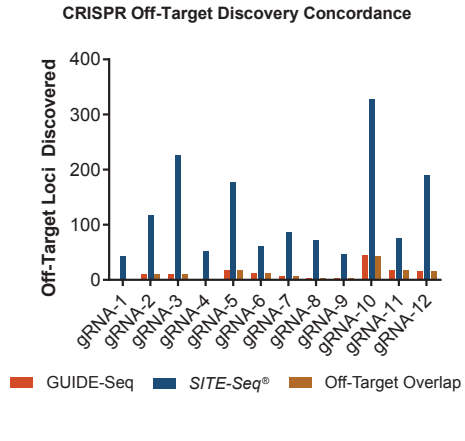
16 nM Cas9 RNP Digestion



64 nM Cas9 RNP Digestion



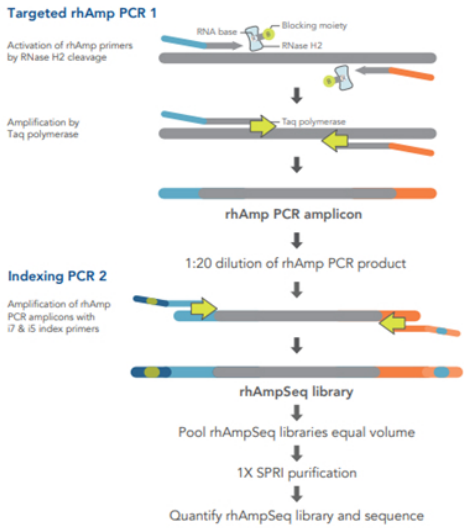
256 nM Cas9 RNP Digestion



Comparison of Off-Target Profiles

The number of potential off-target sites discovered by *SITE-Seq*® increases with Cas9 RNP concentration and is a super-set of the potential off-target loci discovered by GUIDE-Seq.

OFF-TARGET INDEL VALIDATION WITH TARGETED OFF-TARGET SEQUENCING



ID	Top 30 *In silico*	GUIDE-Seq	64 nM RNP *SITE-Seq*®
gRNA-1	0/30	0/2	0/1
gRNA-2	0/30	1/11	1/14
gRNA-3	0/30	1/11	1/27
gRNA-4	0/30	0/0	0/4
gRNA-5	1/30	1/18	2/37
gRNA-6	0/30	0/12	0/3
gRNA-7	0/30	0/7	0/15
gRNA-8	0/30	0/4	0/9
gRNA-9	0/30	1/4	1/4
gRNA-10	0/30	2/45	6/43
gRNA-11	0/30	0/19	1/12
gRNA-12	0/30	0/16	0/31

rhAMPSeq®

RNase H2 dependent PCR Amplification for Next Generation Sequencing (*rhAMPSeq*®), validates off-target editing with multiplexed PCR and targeted off-target sequencing. Table reflects the number of off-target editing loci tested (denominator) and the number of validated off-target (numerator).

CONCLUSIONS

Bioinformatics prediction allowing up to six mismatches and non-canonical PAMs identifies >80,000 potential off-target sites. Therefore, empirical off-target discovery assays facilitate the discovery of potential off-target editing loci for validation and quantification with targeted off-target sequencing in edited cells. The cell-based assay GUIDE-Seq is less sensitive than biochemical off-target discovery assays like *SITE-Seq*® because cell-based assays face DNA editing limitations in a cellular context. This is evident from the data displayed in the above table where some of the validated off-target edited loci discovered by biochemical *SITE-Seq*®, failed to be discovered by the cell-based discovery assay GUIDE-Seq. In addition, all the potential off-target sites discovered by GUIDE-Seq were also discovered by *SITE-Seq*®. Furthermore, cell-based off-target discovery assays are restricted by tissue type and executed in *in vitro* cell culture systems, while biochemical off-target discovery assays are devoid of CRISPR/Cas9 enzymatic restrictions and serve as a species specific off-target discovery assay. Based on the supporting data, it is clear that, cell-based GUIDE-Seq can be deprecated in favor of more sensitive off-target discovery assays like *SITE-Seq*®.

REFERENCES

[1]P Cameron et al. Mapping the genomic landscape of CRISPR-Cas9 cleavage. (2017) *Nature Methods* 14; 600-606.

[2]S Tsai et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. (2015) *Nature Biotech* 33; 187-197.